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Modulation of Cellular Proliferation Alters Glutamine Transport and Metabolism in Human Hepatoma Cells

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Objective
The authors determined the effects of growth inhibition on glutamine transport and metabolism in human hepatoma cells.

Summary Background Data
Hepatoma cells exhibit markedly higher (10- to 30-fold) glutamine uptake than normal human hepatocytes, via a disparate transporter protein with a higher affinity for glutamine. Currently, little is known about the effects of growth arrest on glutamine transport and metabolism in hepatoma cells.

Methods
The authors determined proliferation rates, glutamine transport, and glutaminase activities in the human hepatoma cell lines HepG2, Huh-7, and SK-Hep, both in the presence and absence of the chemotherapeutic agents novobiocin and sodium butyrate. The transport activities for alanine, arginine, and leucine also were determined in both treated and untreated cells. Glutaminase activity was determined in normal human liver tissue and compared with that present in hepatoma cells.

Results
Glutaminase activities were similar in all three cell lines studied, despite differences in proliferation rates, and were sixfold higher than the activity in normal human liver. In contrast to normal hepatocytes, which expressed the liver-specific glutaminase, hepatomas expressed the kidney-type isoform. Sodium butyrate (1 mmol/L) and novobiocin (0.1 mmol/L) inhibited cellular proliferation and reduced both glutamine transport and glutaminase activity by more than 50% after 48 hours in the faster-growing, less differentiated SK-Hep cells. In contrast, the agents required 72 hours to attenuate glutamine uptake by 30% and 50% in the slower-growing, more differentiated HepG2 and Huh-7 cell lines, respectively. Treatment of all three cell lines with novobiocin/butyrate also resulted in a 30% to 60% attenuation of the transport of alanine, arginine, and leucine, and glutamine, indicating that inhibition of cellular proliferation similarly affects disparate amino acid transporters.

Conclusions
Hepatocellular transformation is characterized by a marked increase in glutamine transport and metabolism. Inhibition of cellular proliferation attenuates glutamine transport and metabolism,

especially in fast-growing, relatively undifferentiated hepatoma cells. Because the uptake of other amino acids is similarly reduced under cytostatic conditions, plasma membrane amino acid transport activity in hepatoma cells is regulated by the proliferation state of the cells.

The liver plays a central role in nitrogen metabolism, and performs the energy-dependent conversion of toxic ammonia to urea via the ornithine cycle.[1] Glutamine is the most abundant amino acid in the plasma and serves as the primary nontoxic shuttle for ammonia from extrahepatic tissues to the liver, from which it is taken up via a liver-specific amino acid transporter termed System N.[2] Once inside the parenchymal cell, glutamine is transported across the innermitochondrial membrane into the matrix, where it is hydrolyzed to ammonia and glutamate by a liver-specific glutaminase isozyme.[3] The resulting ammonia is used for urea synthesis, and the glutamate carbon skeleton is used for gluconeogenesis and other metabolic pathways. Net glutamine balance across the liver is achieved by the position-specific expression of glutamine-using (glutaminase/urea synthesis) enzymes in the periportal hepatocytes and glutamine synthetase in a small population of perivenous hepatocytes surrounding the sinusoidal outflow. The simultaneous operation of these pathways results in an intercellular glutamine cycle.[4] The flux through each pathway is highly regulated and determines nitrogen and glutamine balance across the liver and also is implicated in acid/base homeostasis. Thus, two liver-specific proteins, System N and liver-type glutaminase, are expressed by hepatic tissue to support the role of the liver in whole-body nitrogen homeostasis.

In contrast to normal hepatocytes, which are nonproliferative cells, hepatoma cells must obtain increased amounts of nitrogen and carbon to support cellular growth processes. Although tumor cells can use carbohydrate, lipid, or amino acids to obtain energy, glutamine appears to be the preferred substrate and is the main source of nitrogen for purine and pyrimidine biosynthesis.[5] Indeed, the host with cancer becomes progressively glutamine-depleted as tumor growth progresses,[6] which underscores the description of tumors as "nitrogen traps." To gain insights into hepatocellular transformation, we previously examined glutamine transport in normal human hepatocytes isolated from surgical biopsies and found that similar to rat liver, System N accounts for the majority of glutamine uptake.[7] In the same studies, glutamine transport was examined in two human hepatoma cells lines—a well-differentiated hepatoblastoma, HepG2, which retains the expression of most liver-specific genes,[8] and SK-Hep, a fast-growing, poorly differentiated hepatoma cell.[9] The hepatoma cells transported glutamine 10 to 30 times faster than normal parenchymal cells, through a high-affinity carrier determined to be System ASC, although the HepG2 cells also expressed System N. Transport also was examined in fetal human hepatocytes and similar to the adult cells, System N was entirely responsible for glutamine uptake, albeit at a threefold higher rate. The conclusions from these studies were that 1) human liver expresses a unique glutamine transporter (System N) similar to the operative carrier in rat liver, and 2) a high-affinity glutamine transporter (System ASC) is expressed during the process of hepatocellular transformation, the appearance of which is not associated with cell growth per se, because it was not operative in fetal (growing) hepatocytes.

The studies presented here were initiated to determine the effects of attenuation of cellular proliferation on glutamine transport and metabolism in hepatoma cells. In addition to the two cell lines used previously, a third well-differentiated human hepatoma cell,[10] Huh-7, was employed in the investigation. Novobiocin and sodium butyrate, two compounds used by other investigators to arrest cell growth and induce differentiation of hepatoma cells,[11] were chosen to arrest the growth of the three cell lines. Our results indicate that hepatoma cells possess higher glutaminase activity than normal human liver, and that the glutaminase expressed is the "kidney-type" isozyme. The less differentiated, faster-growing hepatoma cell line, SK-Hep, was more sensitive to the growth inhibitory agents than the other two well-differentiated cell lines, and attenuation of cell proliferation in all cell lines resulted in a 30% to 60% decrease in the uptake of glutamine and other amino acids. Collectively, the data indicate that profound metabolic changes occur during hepatocellular transformation, and that the maintenance of enhanced cellular amino acid metabolism is dependent on normal progression through the cell cycle.

METHODS

Hepatoma Cell Culture

The cell line HepG2 was obtained from American Type Culture Collection (Rockville, MD), SK-Hep cells

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were obtained from Dr. Jitka Olander at Monsanto Company (St. Louis, MO), and the Huh-7 cell line was obtained from Dr. Jake Liang at Massachusetts General Hospital (Boston, MA). All tissue culture media, fetal bovine serum, and media additives were purchased from GIBCO BRL/Life Technologies (Grand Island, NY). All tissue culture vessels were obtained from Falcon (Becton Dickinson Inc., Bedford, MA), except for the 24-well culture trays, which were obtained from Costar Corporation (Cambridge, MA). Cells were grown in Dulbecco's Modified Eagle's Media (DMEM) or Dulbecco's Modified Eagle's Medium/Ham's Nutrient Mixture F12 (DME/F12) supplemented with 5% (vol/vol) fetal bovine serum (FBS) and penicillin/streptomycin in 75 cm^2 or 175 cm^2 T-flasks. Cultures were maintained in a humidified atmosphere of 5% CO_2/95% air (NuAire Inc., Plymouth, MN). Cells were passaged by trypsinization on a weekly basis, and the media were replenished every 48 to 72 hours.

For experiments, cells were trypsinized, passaged three times through a 23-gauge needle fitted to a syringe and quantified in a Coulter Electronic Cell Counter (Coulter Electronics, Miami, FL). The desired number of cells then were added to the appropriate volume of culture medium and seeded at the indicated densities in 24-well culture trays (transport studies, 0.5 mL/well), 12-well trays (cell proliferation assays, 1 mL/well), or 100-mm culture dishes (glutaminase assays, 20 mL/dish). All biochemicals used in this study were purchased from Sigma Chemical Company of St. Louis, Missouri.

Amino Acid Transport in Hepatoma Monolayer Cultures

Transport of radiolabeled amino acids was determined by a modification of the cluster tray method[12] originally described by Gazzola et al.[13] All ^3H-labeled amino acids were purchased from Amersham Corporation of Arlington Heights, Illinois. The transport buffers consist of Krebs-Ringer Phosphate Buffer, containing either NaCl and NaH_2PO_4 (NaKRP) or the corresponding choline salts (cholKRP). Thus, Na^+-dependent transport is defined as the transport activity in the presence of Na^+ (NaKRP) minus the activity in the absence of Na^+ (cholKRP). Before the transport assay, the cells in the 24-well trays were rinsed twice with 2 mL/well of 37 C cholKRP to remove extracellular Na^+ and amino acids. The assay was initiated by the simultaneous transfer of 37 C transport buffers (cholKRP or NaKRP) containing 5 μCi/mL of the specified ^3H-labeled amino acid plus 10 μmol/L of the same unlabeled amino acid to each of the 24 wells (0.25 mL/well) via the cluster tray. In the kinetic studies, the concentration of unlabeled glutamine was varied between 10 μmol/L and 5 mmol/L. Osmotic compensation for differences in amino acid content was provided by the addition of equimolar amounts of sucrose to the uptake buffers. The cells were allowed to incubate with the uptake mixtures for 1 minute, after which the buffers were discarded into a dish pan and the cells were rinsed rapidly three times with 2 mL/well of ice-cold NaKRP. After blotting the trays on paper towels, residual moisture was allowed to evaporate from each of the wells, and radioactivity representing transported amino acid was extracted by the addition of 0.2 mL/well of 0.2% (wt/vol) sodium dodecyl sulfate plus 0.2 N NaOH. After a 1-hour incubation, a 0.1-mL aliquot from each well was transferred to a 24-well plate, neutralized with 0.01 mL of 2 N HCl, and 1 mL of Microscint-20 (Packard Co., Meriden, CT) was added to each well. After sealing the plate with plastic film (Top-Seal P, Packard Co.), the plate was analyzed for radioactivity by scintillation spectrophotometry in a Packard Top-Count (Packard Instruments, Meriden, CT). The remaining 0.1 mL of cellular extract was analyzed for protein content by a modification of the Lowry procedure.[14] Transport velocities were calculated with the aid of a computer spreadsheet program (Microsoft Excel) on a Macintosh LCII personal computer, which used specific activities of uptake mixes, radioactive counts, and protein values. Transport values are expressed as nmol of amino acid/mg protein/minute, and are the average of at least three separate determinations (±SD).

Determination of Cellular Proliferation Rates

Hepatoma cells were seeded into 12-well culture trays, typically at a density of 1×10^5 cells/well. After 24 hours, the medium was changed to fresh DMEM or DME/F12 + 5% FBS ± novobiocin and sodium butyrate. Approximately every 24 hours thereafter, the cells in each well were trypsinized, diluted in 0.5 mL of culture media, and passaged five times through a 23-gauge needle to achieve single-cell suspensions for quantification. Complete cellular displacement from the culture surface by trypsinization was verified by examination of each well by light microscopy on a Nikon inverted microscope (Nikon Instruments, Melville, NY). A 0.2-mL aliquot of cell suspension from each well was quantified in a Coulter electronic cell counter (Model ZM, Coulter Electronics). Triplicate wells for each condition were quantified at each time point. Growth rates were calculated by regression analysis via a computer program (Cricket Graph), and are expressed as doubling times in units of hours.

Determination of Glutaminase Activity

Hepatoma cell monolayer cultures grown in 100-mm dishes were rinsed twice with ice-cold NaKRP, and were scraped off the plate in 1 mL of 0.15-mol/L K_2HPO_4 (pH 8.1). The resulting cell suspension was centrifuged at 500 × g for 5 minutes in a refrigerated centrifuge (Sigma Instruments, Osterode am Harz, Germany), and the cell pellet was suspended again in 0.25 mL of ice-cold 0.15 mol/L K_2HPO_4 (pH 8.1). The suspension was frozen in a dry ice/ethanol bath and stored at −80 C until it was used in the enzyme assay. The samples were allowed to thaw on ice and were refrozen once more in a dry ice/ethanol bath before being thawed for use in the assay. Two rounds of freeze/thaw aids in the disruption of the cells and facilitates release of mitochondrial glutaminase into the resulting suspension. Protein assays were performed on all of the samples, and equal amounts of protein were added to each of the reactions. Glutaminase activity was determined according to the method of Heini et al.[15] via the determination of ammonia generated with the o-phthaldialdehyde reagent. We have modified this method by reducing the volume of detection reagents tenfold, allowing the assay to be read in a 96-well microplate format through the use of a plate reader (Anthos Labtech Inc., Frederick, MD). Thus, many samples can be read simultaneously. Briefly, 25 μL of cellular extract was added to 35 μL of reaction buffer (0.15 mol/L K_2HPO_4 [pH 8.1], 171 mmol/L L-glutamine, 1 mmol/L NH_4Cl). The samples were incubated for 60 minutes at 37 C, and the reactions were terminated by the addition of 10 μL of ice-cold 10% (wt/vol) trichloroacetic acid. After allowing the tubes to incubate on ice for 15 minutes, the samples were centrifuged in a microcentrifuge for 5 minutes (approximately 12,000 × g), and 5 μL aliquots of the resulting supernatants were analyzed for ammonia content by the addition of 0.15 mL of detection reagent (10 mL 0.2 mol/L K_2HPO_4 [pH 7.4], 0.56 mL 72 mmol/L mercaptoethanol in ethanol, 0.56 mL o-phthaldialdehyde in ethanol). The trays were shaken briefly, and the colorimetric reaction proceeded in the dark for 45 minutes. The absorbance at 405 nm of each well was determined on an Anthos plate reader, and values for ammonia were calculated by the instrument, based on a standard curve of NH_4Cl in the same tray. Blanks from each sample were generated by the addition of 25 μL of cell extract to 35 μL of reaction buffer followed by immediate neutralization with 10% trichloroacetic acid, and were subtracted from the final values for each reaction. Glutaminase activity is presented as μmol NH_3/mg protein/hour, and is the mean ± SD of at least three separate determinations for each sample.

Isolation of Rat Hepatocytes

Rat hepatocytes were isolated from the liver of a male Fisher 344 rat (200 g) by the collagenase procedure described in detail by Kilberg.[12] After filtration of the digested liver tissue over two-ply gauze, the cell suspension was centrifuged at 50 × g for 2 minutes in a refrigerated centrifuge (Beckman Instruments, Palo Alto, CA). The cell pellet contained the parenchymal cells, and the supernatant contained the nonparenchymal cells (Kupffer cells, endothelial cells). The pellet was washed three more times with ice-cold buffer, and the supernatant was centrifuged at 500 × g for 5 minutes to sediment the nonparenchymal cells. After two additional washes of the nonparenchymal cells, the suspension was examined by light microscopy and essentially found to be devoid of parenchymal cells (3% to 4% contamination). Aliquots of 10^7 cells were centrifuged, and the cell pellets were suspended again in 0.5 mL of 0.15 mol/L K_2HPO_4 (pH 8.1), frozen, and stored at −80 C until they were used in the aforementioned glutaminase assay.

Preparation of Human Liver Extract

A small wedge biopsy (approximately 2 g), obtained from a 27-year-old female patient with normal liver function tests, was dropped immediately into liquid nitrogen. The biopsy was stored under liquid nitrogen until prepared for use in the glutaminase assay described. Briefly, a small piece of the biopsy was obtained and pulverized with a mortar and pestle on dry ice. The resulting powder was transferred to a Dounce homogenizer containing 2 mL of ice-cold 0.15 mol/L K_2HPO_4 (pH 8.1), and was subjected to ten strokes with a loose-fitting pestle and ten strokes with the tight-fitting pestle. The homogenate was transferred to two 1.5-mL centrifuge tubes and stored at −80 C until used in the glutaminase assay.

Statistical Analyses

Differences in measured parameters between cells treated with different experimental conditions were analyzed statistically by Student's t test, and a p value of <0.05 was considered significant.

RESULTS

Glutaminase Activity in Hepatoma Cells and Normal Liver Cells

Two types of phosphate-dependent glutaminase are expressed in mammalian tissues—the "kidney-type" found in most tissues, and the "liver-type," which is liver-specific and is linked functionally to the urea cycle.[3] Studies with rat hepatoma cells showed that, on cellular transformation, the liver-type isozyme is lost and is replaced by the kidney-type.[16] To determine if the same

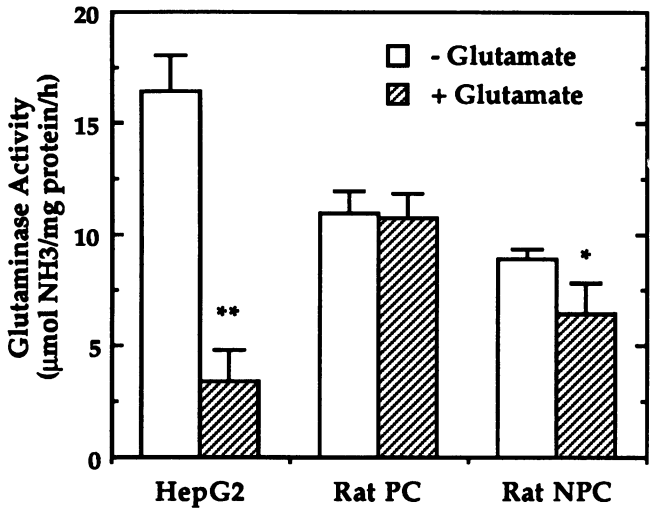

Figure 1. Effect of glutamate on phosphate-dependent glutaminase activity in HepG2 cells and rat liver cells. HepG2 cells, rat liver parenchymal cells (Rat PC) and rat liver nonparenchymal cells (rat NPC) were isolated and prepared for the glutaminase assay as described in the Methods section. Equal amounts of cellular protein were added to the reaction buffer, which contained 171 mM of L-glutamine ± 171 mM of L-glutamate. This level of glutamine commonly is used to measure the activity of the liver-type isozyme. After 30 minutes at 37 C, the reaction was terminated, and the NH_3 content of each sample was determined with o-pthaldialdehyde/mercaptoethanol solution. The data are presented as μmol NH_3 produced/mg protein/hour, and are the averages ± SD of three separate determinations. Only the kidney-type glutaminase isozyme is subject to inhibition by its product glutamate. (**$p < 0.01$, *$p < 0.05$ compared with values obtained in the absence of glutamate).

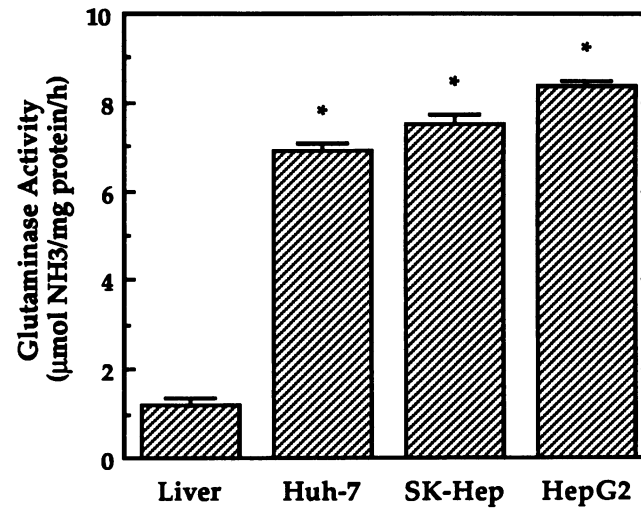

Figure 2. Comparison of glutaminase activity in human hepatoma cells and human liver. Hepatoma and human liver homogenates were prepared for the glutaminase assay as described in the Methods section. Equal amounts of protein (85 μg) diluted to a final volume of 25 μL were added to 35 μL of the reaction buffer, which contained 24 mM of L-glutamine (a concentration sufficient for measurement of the kidney-type isozyme). The reaction was allowed to proceed for 60 minutes at 37 C before termination by the addition of trichloroacetic acid. Previous studies showed that the production of NH_3 was linear over time from 0 to 60 minutes (data not shown). The data presented as μmol NH_3 produced/mg protein/min and are the averages of three separate determinations ± SD (**$p < 0.01$ compared with activity in human liver, which was 1.19 ± 0.17).

phenomenon occurs in human hepatoma cells, glutaminase activity was examined in the very well-differentiated cell line HepG2 and compared with that present in rat parenchymal and nonparenchymal cells. The results presented in Figure 1 demonstrate that 80% of glutaminase activity in the HepG2 cells was inhibited by the presence of equimolar amounts of glutamate. Because only the kidney-type isozyme is sensitive to inhibition by its product, glutamate,[3] these results indicate that a loss of the liver-specific glutaminase isozyme and appearance of the kidney-type isozyme also are associated with hepatocellular transformation in human cells. In contrast, glutaminase activity in rat parenchymal cells was unaffected by the presence of glutamate, whereas the activity in nonparenchymal cells was inhibited by approximately 30%. To determine the levels of glutaminase activity in hepatoma cells relative to that present in human liver, cellular extracts from all three cell lines (HepG2, SK-Hep, and Huh-7) and homogenates from a surgical liver specimen were assayed for the production of NH_3 from glutamine. The results, presented in Figue 2, reveal that glutaminase activity is approximately equal in all three cell lines, whereas the activity present in the human liver was only 15% to 18% of the levels found in the hepatomas. Collectively, the data suggest that human hepatoma cells contain markedly higher glutaminase activity than normal human liver, and that this enhanced activity is attributable to the expression of the kidney-type isozyme.

Effect of Novobiocin and Butyrate on Cellular Proliferation

Treatment of Chang liver cells with 0.1 mmol/L of novobiocin, a topoisomerase inhibitor, and 1 mmol/L of sodium butyrate, a short-chain fatty acid, previously was shown to inhibit cellular proliferation and induce a more differentiated phenotype.[11] Through a mechanism that involved the hyperacetylation of nuclear proteins, these agents additively arrested cells in the G_2 stage of the cell cycle. To determine if these agents exert similar effects on the cell lines in the present study, cellular proliferation rates were determined for each of the three hepatomas in the presence or absence of 0.1 mmol/L of novobiocin, 1.0 mmol/L of sodium butyrate, alone or in combination. As illustrated in Figure 3, both novobiocin and butyrate alone slowed the growth rate of all three hepa-

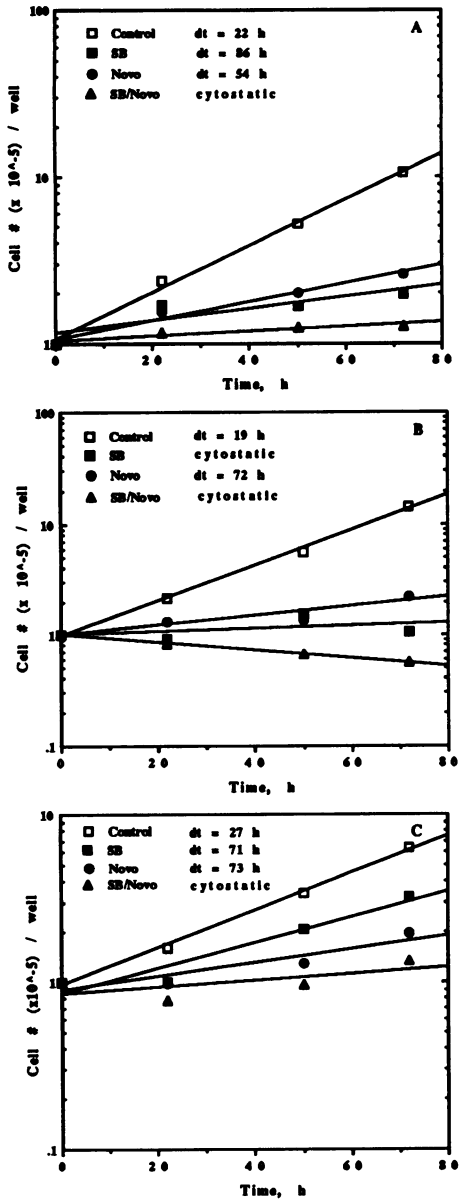

Figure 3. Effect of 0.1 mmol/L of novobiocin and 1.0 mmol/L of sodium butyrate on cellular proliferation of human hepatoma cells. Cells were seeded at an initial density of 1×10^5 cells/well in a 12-well culture dish. After 24 hours, the medium was changed to fresh DME/F12 + 5% FBS ± sodium butyrate (SB), novobiocin (Novo), or both (SB/Novo). Every 24 hours thereafter, cells were quantified in a Coulter electronic cell counter. (A) HepG2; (B) SK-Hep; (C) Huh-7. Doubling times (dt) are indicated. Each point represents the average of triplicate determinations. Standard deviations, which typically were less than 10%, are omitted for clarity.

toma cells, but in combination, inhibited proliferation completely. The inhibitory effects of the agents on cellular growth are rapid (i.e., they occur during the first 24 hours); no "lag phase" was apparent before growth arrest was achieved. To ensure that the cells were still viable after treatment with these compounds, cell viability was determined after 72 hours of incubation by trypan blue exclusion. In all three hepatomas, viability remained greater than 95% after growth arrest, and the actual cell viabilities were as follows: HepG2, 95.3 ± 0.5%; SK-Hep, 99.7 ± 0.6%; Huh-7, 98.8 ± 1.1%. The data demonstrate that treatment of hepatoma cells with a combination of 1.0 mmol/L of sodium butyrate and 0.1 mmol/L of novobiocin results in growth arrest of the cells without compromising cellular viability, consistent with observations in studies with other hepatoma cells.[11]

In addition to acting as cytostatic agents, novobiocin- and butyrate-induced profound morphologic changes in the hepatoma cells to a more fibroblastic phenotype. These changes in morphology were more pronounced and occurred more rapidly in the SK-Hep cells than in the Huh-7 or HepG2 (Fig. 4). Changes in cellular morphology induced by novobiocin and butyrate were evident as early as 24 hours after addition of the agents in SK-Hep cells, whereas the slight changes in phenotype in the Huh-7 and HepG2 cell lines required 48 to 72 hours to become noticeable. Previous work demonstrated that increases in cytoskeletal protein synthesis accompanied this switch to a fibroblastic morphology.[11]

Effect of Butyrate and Novobiocin on Hepatoma Amino Acid Transport

Because the agents completely arrest the growth of cells, the effects of cell density on glutamine transport had to be addressed to ensure that changes in cellular metabolism elicited by the agents were not simply a result of differences in the degree of confluence. Each of the three hepatoma lines was seeded into 24-well trays at specific cell densities from 5×10^4 cells/well to 3×10^5 cells/well and allowed to incubate for 24 hours. The Na^+-dependent uptake of 10 μmol/L of L-glutamine then was examined, and the results are presented in Figure 5. The results indicate that Na^+-dependent glutamine transport activity is higher in more confluent cultures, especially in SK-Hep and Huh-7 cells, in which there was a twofold difference ($p < 0.01$) in uptake between the lowest and highest plating densities. Once a given cell density was reached (1×10^5/well for HepG2 and SK-Hep, and 2×10^5/well for Huh-7), further increases failed to affect transport. Based on this information, in all subsequent experiments, cells that were to receive treatment with butyrate/novobiocin were plated at twofold higher densities than control cells, to ensure that after 48 to 72 hours of treatment, the number of cells in each well would be approximately equal. In addition to providing information on density effects, Figure 5 also illustrates the differences in glutamine transport activity between the

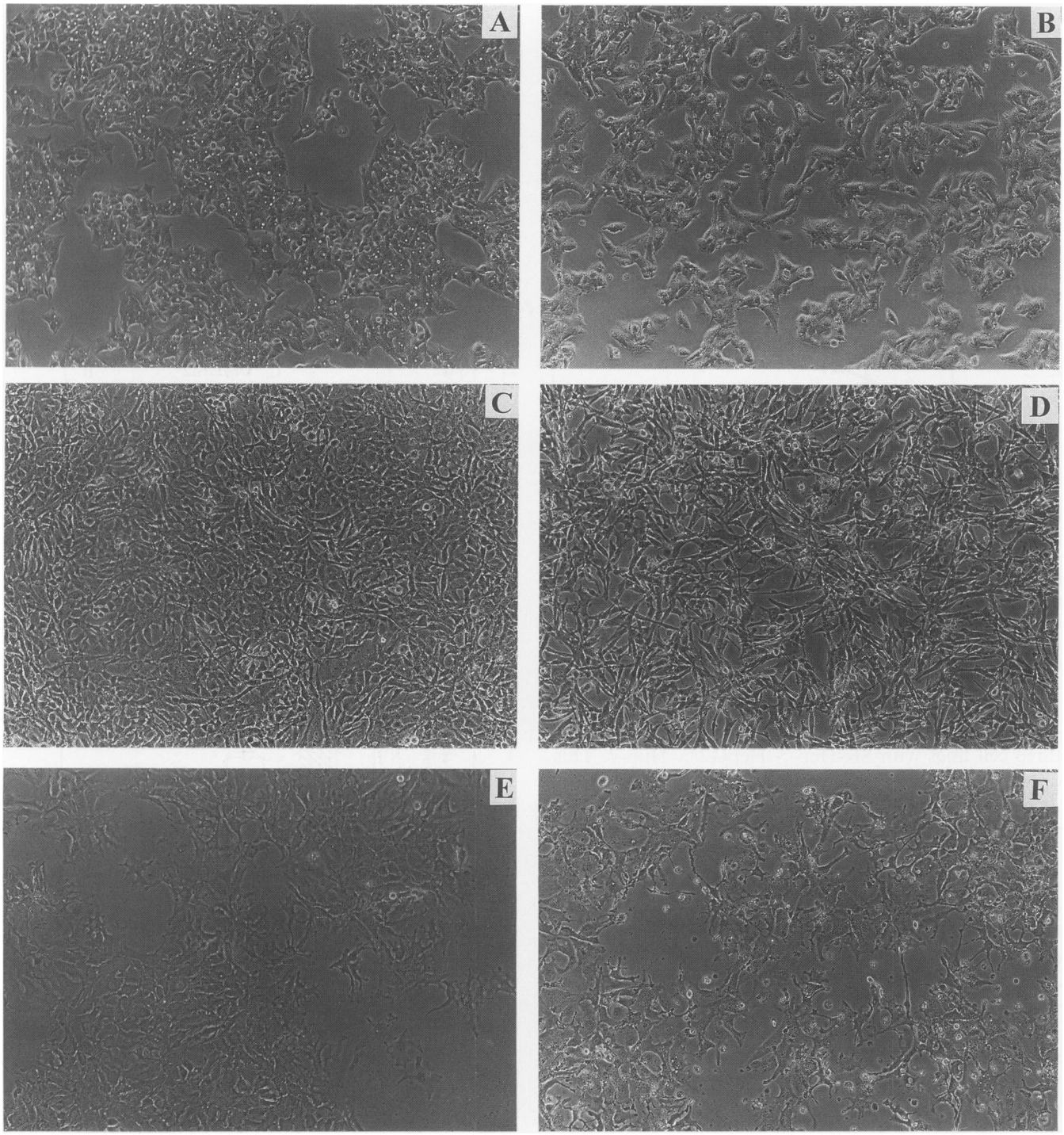

Figure 4. Effect of novobiocin and butyrate on cellular morphology. Hepatoma cells were cultured in the absence (control) or presence of 0.1 mmol/L of novobiocin and 1.0 mM of butyrate (SB/Novo) in DME/F12 + 5% FBS for 48 hours. All photographs are 100 × magnification. (A) HepG2 control; (B) HepG2 + SB/Novo; (C) SK-Hep control; (D) SK-Hep + SB/Novo; (E) Huh-7 control; (F) Huh-7 + SB/Novo. The level of confluence of the cell lines underscores faster growth rates of the SK-Hep (C) relative to the HepG2 and Huh-7 cells, because cells initially were seeded at the same density (5×10^4 cells/cm^2). Note also the dramatic shift to a fibroblast-like morphology in the SK-Hep cells (D) relative to the other cell lines.

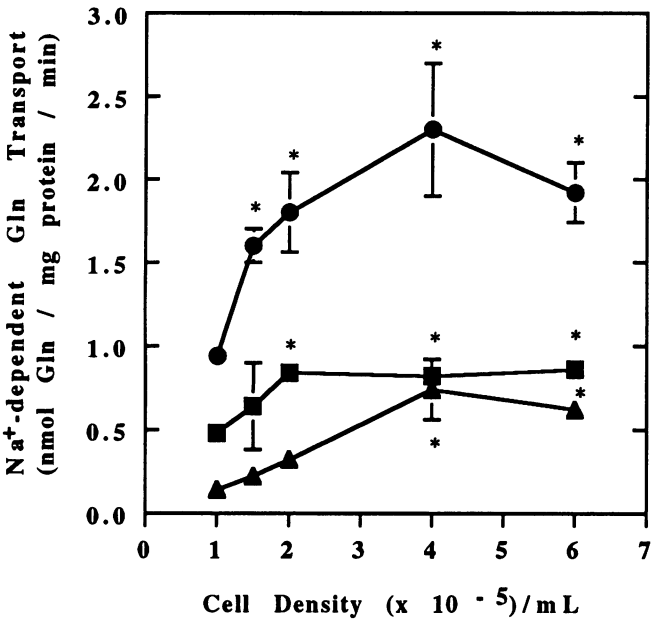

Figure 5. Effect of cell density on Na^+-dependent glutamine transport activity. Human hepatoma cells initially were seeded at the indicated cell densities into 24-well culture plates (0.5 mL/well). After a 24-hour incubation, the Na^+-dependent uptake of 10 μM of L-glutamine was monitored for 1 minute at 37 C. Each point represents the average ± SD for quadruplicate determinations. This figure also illustrates the relative rates of glutamine uptake in the three cell lines studied, with velocities in SK-Hep > HepG2 > Huh-7. (*$p < 0.050$ relative to the transport activity for the lowest cell density [n = 4]).

three cell lines with SK-Hep > HepG2 > Huh-7. This order of decreasing glutamine transport activity relates nicely to the doubling times illustrated in Figures 3A through 3C.

To determine the effects of cell growth arrest on glutamine transport and metabolism, each of the cell lines was cultured in the presence or absence of 1.0 mmol/L of butyrate + 0.1 mmol/L novobiocin for 48 hours. Na^+-dependent glutamine uptake was determined at two different concentrations, 10 μmol/L and 600 μmol/L, which allowed the evaluation of both high-affinity glutamine transport activity and the capacity to transport the amino acid at physiologic levels, respectively. In a parallel set of 100-mm culture dishes, cells cultured ± butyrate/novobiocin were harvested and prepared for use in the determination of glutaminase activity. The results, depicted in Figures 6A through 6C reveal that after 48 hours of treatment, glutamine transport and metabolism were inhibited only in the SK-Hep cell line, in which transport of 10 μmol/L of glutamine, 600 μmol/L of glutamine, and glutaminase activity were inhibited by 56%, 49%, and 64%, respectively. In contrast, glutamine uptake at both concentrations and glutaminase activity were unaffected in HepG2 and Huh-7 cells. These results

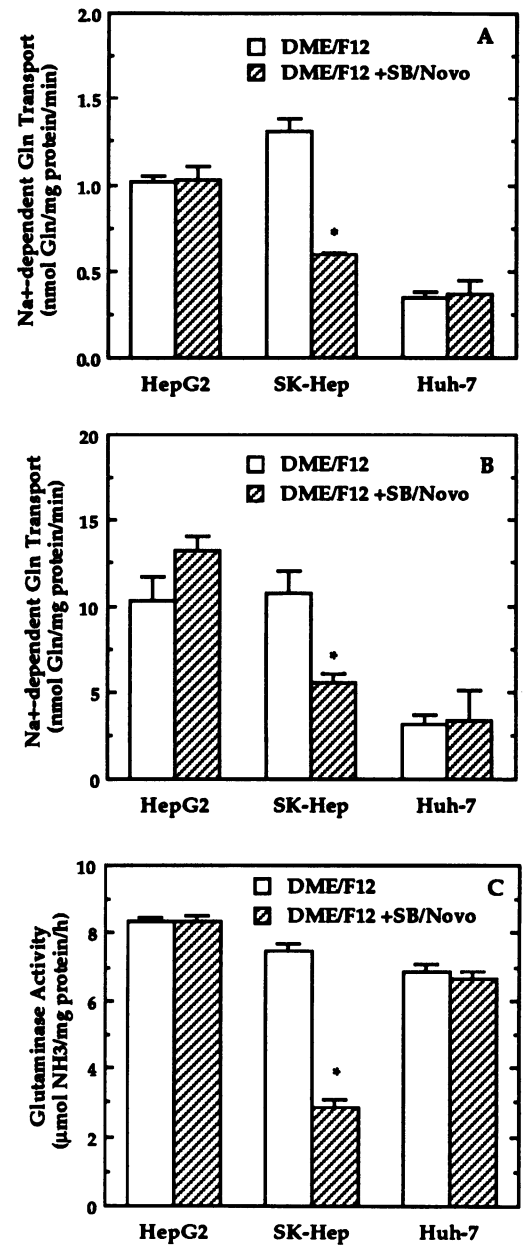

Figure 6. Effect of novobiocin and butyrate treatment on glutamine transport and glutaminase activity after 48 hours. Hepatoma cells were seeded at a density of 5×10^4 cells/well or 1×10^5 cells/well in 24-well trays in DME/F12 + 5% FBS. After 24 hours, the medium was changed to fresh DME/F12 + 5% FBS on the lightly seeded cells or fresh media + 0.1 mmol/L of novobiocin and 1.0 mmol/L of sodium butyrate (+SB/Novo) on the heavily seeded cells. The differential seeding densities were designed to compensate for the altered growth rates caused by the agents. After 48 hours, the Na^+-dependent uptake of either (A) 10 μmol/L or (B) 600 μmol/L of L-glutamine was determined. (C) In a parallel set of 100-mm culture dishes, hepatoma cells treated ± SB/Novo in DME/F12 + 5% FBS for 48 hours were harvested, and glutaminase activity was determined. This figure demonstrates the greater sensitivity of the tumorigenic SK-Hep cell line to the effects of the agents relative to the other two well-differentiated cell lines. All values represent the mean ± SD of triplicate determinations. (*$p < 0.010$ relative to control values [n=3]).

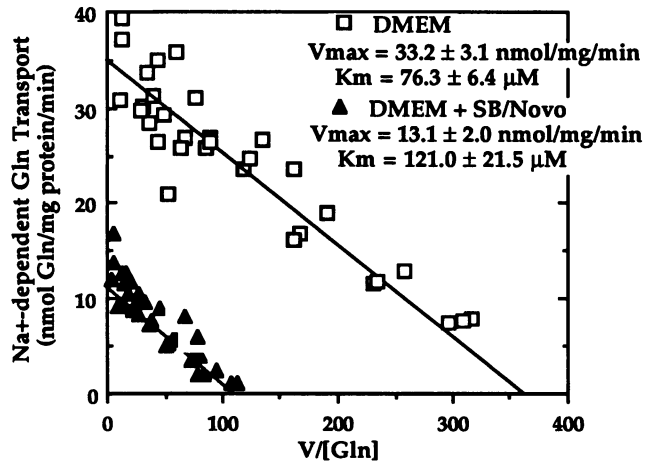

Figure 7. Kinetics of Na^+-dependent glutamine transport in SK-Hep cells after 72 hours of treatment ± butyrate/novobiocin. SK-Hep cells were seeded into 24-well culture trays at either 1.2×10^5 or 0.3×10^5 cells/well. After 24 hours, the medium was changed to DMEM + 5% FBS in the less dense wells or DMEM + 5% FBS + 1.0 mmol/L sodium butyrate and 0.1 mmol/L novobiocin (SB/Novo) in the wells with higher cell densities. Differential seeding densities were employed to compensate for differences in growth rates after addition of the inhibitory agents. After 72 hours of additional culture, the Na^+-dependent transport of glutamine at specific concentrations from 10 μM to 5 mM was monitored for 1 minute at 37 C. The resulting Eadie-Hofstee plot is shown and illustrates that the decrease in glutamine uptake elicited by growth arrest of the cells is caused by a 61% decrease in the maximum velocity (V_{max}) of the transporter ($p < 0.010$ [n = 3]) relative to only a minor change in transporter affinity for glutamine. The kinetic constants are listed in the figure. Each point represents a single determination, and kinetic constants were calculated based on three separate determinations for each condition.

suggest that the faster-growing, less differentiated SK-Hep cells are more susceptible to the cytostatic effects of butyrate/novobiocin. The temporal effects of these agents on cellular metabolism in the SK-Hep cells correlate well with those on cellular morphology.

The kinetic characterization of the inhibitory effects of butyrate/novobiocin on SK-Hep glutamine transport was performed, and the results are presented in Figure 7. After 72 hours of treatment with the agents, Na^+-dependent glutamine uptake was further reduced compared to the 48-hour time point; a 75% reduction in activity was observed for a substrate concentration of 10 μmol/L. As displayed in Figure 7, a 61% decrease in the maximum velocity of System ASC was observed in response to butyrate/novobiocin treatment (33.2 ± 3.1 *vs.* 13.1 ± 2.0 nmol L-glutamine/mg protein/min, for control and SB/Novo, respectively), relative to a small decrease in the affinity for glutamine (Km = 76.3 ± 6.4 *vs.* 121 ± 21.5 for control and SB/Novo, respectively), implying that the number of functional transporters in the plasma membrane was reduced.

To determine if similar effects on transport occur in HepG2 and Huh-7 cells after longer exposures to butyrate/novobiocin, all three cell lines were treated with the agents for 72 hours, and the effects on the transport of four different amino acids was examined. The results, presented in Figure 8, reveal that transport is affected in all three cell lines after 72 hours of treatment. Glutamine transport was reduced by 30%, 73%, and 40% in HepG2, SK-Hep, and Huh-7 cells, respectively ($p < 0.050$ [n = 3]). Similarly, Na^+-dependent alanine transport via System ASC was reduced 25%, 75%, and 36% in HepG2, SK-Hep, and Huh-7, respectively ($p < 0.050$ [n = 3]). In all three cell lines, very little System A activity was observed under either condition, which is not unexpected because the cells are not extensively depleted of amino acids before the transport assay, and thus, this carrier, if present, remains largely undetectable because of transinhibition of activity.[17] System y^+, which mediates the majority of arginine uptake in these cells, also was downregulated in all three cell lines by treatment with butyrate/novobiocin. Reductions in activity of 32%, 65%, and 45% were observed in HepG2, SK-Hep and Huh-7, respectively ($p < 0.050$ [n = 3]). System L, an Na^+-independent carrier that mediates most of the leucine uptake in these cell lines, exhibited the highest activity of any of the transporters examined, and also was diminished by treatment with the agents. In HepG2, SK-Hep, and Huh-7 cell lines, its activity was attenuated by 60%, 39%, and 73%, respectively. Collectively, these data suggest that attenuation of amino acid transport via inhibition of cellular proliferation is not restricted to glutamine, but involves global changes in amino acid transport activities, and that these cytostatic agents require longer periods of time to elicit changes in the cellular metabolism of well-differentiated hepatoma cells.

DISCUSSION

The process of tumorigenesis is associated with a marked increase in cell proliferation and a simultaneous increase in the uptake of glutamine to support energy metabolism and nucleic acid synthesis. Previous work from our laboratory characterized glutamine transport in normal human liver parenchymal cells and in the cell lines SK-Hep and HepG2.[7] In those studies, it was shown that hepatoma cells transport glutamine 10 to 30 times faster than human hepatocytes. Kinetic analysis revealed that the basis for this augmented transport capacity was the high-level expression of a high-affinity transporter for glutamine that is not found in normal hepatocytes. Amino acid inhibition analysis revealed this transporter to be System ASC, similar to the one reported in fibroblasts,[18] but distinct from the liver isoform.[19] Those studies also established that both adult and fetal hepatocytes

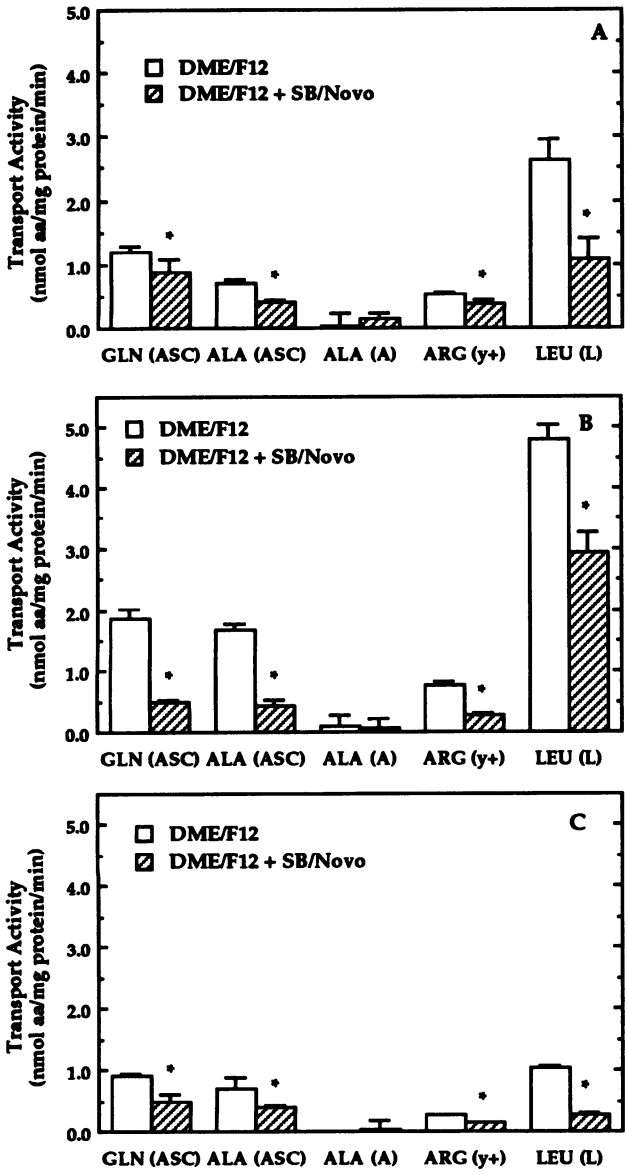

Figure 8. Effect of sodium butyrate/novobiocin treatment for 72 hours on amino acid transport systems in hepatoma cells. Hepatoma cells were seeded initially at low (5×10^4 cells/well) or high (1×10^5 cells/well) densities in 24-well trays. After 24 hours, the medium was changed to DME/F12 + 5% FBS (low density cultures) or the same medium + 1.0 mmol/L of sodium butyrate and 0.1 mmol/L of novobiocin (SB/Novo) in the higher cell density wells. The cells were allowed to incubate an additional 72 hours, at which point the uptake of specific amino acids at 10 μmol/L was monitored for 1 minute at 37 C. The activity of individual transport systems was determined as follows: System ASC, Na^+-dependent alanine uptake not inhibited by excess (5 mmol/L) MeAIB; System A, Na^+-dependent alanine transport inhibited by 5 mmol/L MeAIB; System y^+, saturable, Na^+-independent arginine uptake; and System L, saturable Na^+-independent leucine uptake inhibited by excess (25 mmol/L) BCH. The transport system for each set of data is indicated in parentheses. The y-axis scales are the same in all graphs to facilitate the comparison of amino acid transport activities between cell lines: (A) HepG2; (B) SK-Hep; (C) Huh-7. MeAIB = 2-(methylamino)-isobutyric acid, BCH = 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid. (*$p < 0.050$ compared with control (untreated) transport values. [n = 3]).

use System N, which has a Km for glutamine of approximately 500 μmol/L. Fetal hepatocytes exhibited approximately threefold higher glutamine transport activity than the adult cells. The observation that fetal hepatocytes, cells that are engaged actively in growth and cell cycling, did not express the System ASC found in tumor cells suggested that expression of this ASC isoform is not associated with cell growth per se, but may be expressed as part of cellular transformation, or dedifferentiation (e.g., fibroblasts), a process often associated with malignancy. Finally, it was established through kinetic analysis that the well-differentiated HepG2 cell retained expression of System N and ASC, whereas SK-Hep cells expressed only System ASC for the uptake of glutamine.

The studies presented here established that although HepG2 cells are well differentiated, the kidney-type glutaminase isozyme is primarily operative in these cells (Fig. 1) and that glutaminase activity, similar to glutamine transport, is elevated in human hepatoma cells compared with the activity present in normal liver (Fig. 2). Figure 9 summarizes the differences in glutamine transport and metabolism between human hepatocytes and hepatoma cells. Because hepatoma cells are engaged in active growth, they express two disparate proteins designed to fulfill the heightened demands of the cell for glutamine—namely System ASC and kidney-type glutaminase. These two proteins possess higher affinities for glutamine than the normal hepatocyte counterparts[3,7] (System N and liver-type glutaminase), which ensures that hepatoma glutamine metabolism always operates at maximum rates, regardless of changes in plasma glutamine levels. Indeed, it is well-established that tumor cells use far more glutamine than mandated by their metabolic needs.[5,6] The central question raised by our research endeavor is: What are the cellular signals responsible for the expression of System ASC and kidney-type glutaminase during hepatocellular transformation? As a first step in the elucidation of the pathways involved, the relationship between cellular proliferation and the activity of System ASC was examined.

Figure 3 illustrates that novobiocin and sodium butyrate alone each significantly attenuate proliferation in all three cell lines, but in combination, inhibit proliferation almost entirely. The same observation was made in Chang liver cells,[11] in which these agents were shown—in combination—to arrest cells in the G_2 phase of the cell cycle and induced a more differentiated phenotype. The

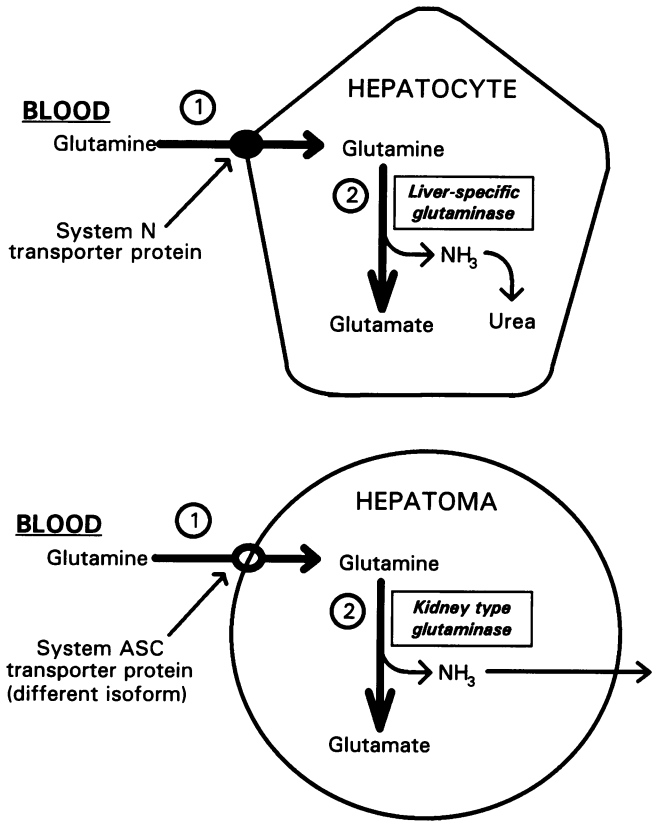

Figure 9. Characteristics of glutamine metabolism in normal hepatocytes and hepatoma cells. Transport across the plasma membrane and inner mitochondrial membrane and hydrolysis of glutamine by phosphate-dependent glutaminase in the mitochondria represent the first two steps in the control of cellular glutamine metabolism. Most glutamine metabolism in the liver is linked to urea synthesis, whereas glutamine metabolism in tumor cells is geared toward oxidative metabolism. Hepatocellular transformation results in the expression of a high-affinity glutamine transporter, System ASC, which is distinct from the System ASC isozyme in normal liver, which does not transport glutamine. Likewise, the liver-type glutaminase is replaced by the kidney-type glutaminase, which possesses a higher affinity for glutamine. Based on data presented in this report, it appears that the level of activity of these two proteins (System ASC and glutaminase) is linked to continuous progression through the cell cycle.

effects of these agents on proliferation appeared to occur during the first 24 hours of treatment, based on the growth curves presented in Figure 3; however, the effects on transport were much more rapid in SK-Hep cells than in the other two cell lines (Figs. 6A and 6B) and were observable as early as 24 hours after initial treatment (data not shown). This observation may relate to the clinical observation that faster growing tumors respond better to chemotherapeutic agents than slower growing tumors. In fact, the agents elicited both metabolic (Fig. 6C) and morphologic effects (Fig. 4) on SK-Hep cells much more rapidly than on the other more differentiated cell lines, which required exposure times of 72 hours before any effects on transport were observed (Figs. 8A and 8C). given that cell growth is almost totally arrested after just 24 hours, this suggests that factors controlling amino acid transport activity may be affected "downstream" after arrest of cellular proliferation. Genes associated with liver cell growth, such as c-myc,[20] may participate in the regulation of activity of transporters. We currently are initiating studies to address this possibility.

Based on the data in Figure 8, it appears that glutamine metabolism is not inhibited selectively by cytostatic agents, but rather, a global downregulation of amino acid transport activity was elicited. Clearly, other amino acid transport activities are elevated in the hepatoma cells besides glutamine. For example, arginine transport via System y^+ was increased 15-fold in SK-Hep (0.77 nmol/mg protein/min), 10-fold in HepG2 (0.53 nmol/mg protein/min), and 5-fold in Huh-7 cells (0.26 nmol/mg protein/min) *versus* the activity we observed in human hepatocytes[21] (0.05 nmol/mg protein/min).

As a first step in the attempt to elucidate the events that are linked to the expression of System ASC and kidney-type glutaminase during hepatocellular transformation, these studies confirmed that human hepatoma cells express the kidney-type glutaminase isozyme, and that the level of activity of System ASC and other amino acid transporters is linked to the proliferation state of the cell. We currently are initiating studies to address the role of certain oncogenes, such as myc[20] and ras,[22] in the induction of System ASC and the differential expression of glutaminase isozymes during hepatocellular transformation.

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Discussion

DR. SAMUEL A. WELLS, JR. (St. Louis, Missouri): This is an excellent paper, which represents experiments carefully done by an outstanding investigator who has made several contributions to the study of this transport mechanism both in the normal and the diseased state. I would like to ask Dr. Souba and his associate three questions.

The first is, what happens to all the glutamine? Is it utilized by the cell or is there excess glutamine? This seems an important consideration since these tumor cells seem to serve as nitrogen traps, often to the detriment of the rest of the body.

Have you had a chance to study hepatic adenoma cells? They represent a cell system along the spectrum from fetal cells to normal liver cells to well-differentiated and undifferentiated malignant cells. Do they have a different metabolic spectrum?

The most intriguing matter of this paper seems to be the ACS transport system. Does it have a counterpart in other cell systems, in other malignancies? Does the author have any insight into the regulation of the system? It seems perhaps to represent an important point for therapeutic intervention if its mechanism of action is clearly understood.

DR. MURRAY F. BRENNAN (New York, New York): I thank Dr. Souba for asking me to comment on the paper. The utilization of substrate and protein synthetic activity in hepatocytes is a very complicated issue.

We have previously shown in human liver biopsies that the rate of protein synthesis is influenced by such diverse factors as antecedent weight loss in the patient, the presence of a remote malignancy, and indeed exaggerated in the normal liver by the presence of an intrahepatic metastasis. Dr. Souba has shown that in isolated cell systems malignant hepatic cells have an accelerated rate 10 to 20 times that of normal fresh hepatocytes.

This is a difficult subject to study. The appropriate control cell is very difficult to establish. The active tumor cells are actively dividing cells, whereas the normal hepatocytes are rather quiescent. The first question then, can the data be explained solely on the increased metabolic rate of a dividing cell?

In the manuscript the authors refer to unpublished work that compares these malignant cells to fetal human hepatocytes, where the rate, as you saw, was threefold increased over normal liver. But again, fetal cells may be growing but not necessarily dividing, and certainly not necessarily dividing at the same rate as the malignant cells.

The key observation, however, is that the way in which the malignant cells transport glutamine is clearly changed. It's not just that they try harder, they do seem to have "the malignant advantage." Then the control becomes not just dividing cells but other malignant cells. So the second question is, do other malignant cells have an accelerated transport based on this new ASC transporter?

And the third question, what turns this process on? And the fourth, as Dr. Wells suggested, where does the glutamine go? Is it used for energy? Do such rapidly dividing cells just deplete glucose and turn to an alternative energy source?

Finally, the butyrate and novobiocin seems, Dr. Souba, to act more as a differentiation agent. Any information that leads to a therapeutic strategy is desperately needed for hepatoma, given our current success.

DR. JOSEF E. FISCHER (Cincinnati, Ohio): This is an excellent study very difficult to present because of its complicated nature and I congratulate Dr. Souba on its presentation.

Several years ago a number of laboratories, including our own, noted that you could tell the size of the tumor and its metabolic activity as it was inversely related to the serum level of glutamine. And not only glutamine was affected in this way, but alanine and serine, two gluconeogenic acids.

About a year later Dr. Souba's lab presented, I believe at the Surgical Forum, a very intriguing paper which demonstrated that in animals with tumors as the extraction of glutamine increased the extraction by the gut decreased and they seemed to be inversely related. And subsequently, Dr. Norton's lab, our